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Enzymatic Determination of Glycerol and Glyceride-Glycerol in Plasma

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Die von Wieland beschriebene Methode für die enzymatische Glycerinbestimmung im Plasma wurde im Enteiweißungsverfahren, der Reihenfolge der Zugabe der Reagenzien und der Zeit der Extinktionsmessung vereinfacht. Die Methode wurde für die Bestimmung von Glycerid-Glycerin im Plasma erweitert.

The method described by Wieland for the enzymatic determination of glycerol in plasma was simplified in the deproteinization procedure, the sequence of adding the reagents and the time of reading the optical density. The method has been extended to the determination of glyceride-glycerol in plasma.

The enzymatic determination of glycerol has been elaborated by Wieland (1, 2) and modified by Spinella and Mager (3). The latter authors extended the method to the determination of glyceride-glycerol, Glycerol is phosphorylated to L(-)-glycerol-1-phosphate by ATP and GK[1]) (2, 4). Under the influence of α-GDH, glycerol-1-phosphate and NAD are converted into DOAP and NADH (5).

An alkaline medium with an excess of NAD is used to increase the equilibrium constant of this reaction.

Finally DOAP is trapped by hydrazine (4, 6).

The optimal pH for enzyme activity of glycerolkinase is 9,8; Mg^{++} is needed as an activator (4).

We modified the method of Wieland and Spinella-Mager as follows:

1. deproteinization by the Somogyi procedure (7).
2. except for GDH, all reagents were added to the filtrate as a mixture and not separately.
3. the increase of optical density was measured once, one hour after adding GDH, and not at short intervals until a maximum value was attained.

[1]) *Abbreviations:* GK = glycerolkinase (ATP: glycerol phosphotransferase EC 2.7.1.30); α-GDH = Glycerolphosphate dehydrogenase (L-glycerol-3-phosphate: NAD Oxidoreductase EC 1.1.1.8); DOAP = dihydroxy-acetone-phosphate.

[2]) Both reagents are to be titrated one against the other.

[3]) Boehringer Biochimica.

Methods

Reagents

Zinc sulphate 0.25N[2])
Sodium hydroxide 0.25N[2])
Buffer: glycine 0.2M; hydrazine 1M; Mg^{++} $2 \cdot 10^{-3}$M, pH 9.8 Store at 4° C in a brown bottle and prepare fresh every 4 weeks.
ATP[3]) 0.05M
Neutralised with 1N NaOH, the solution is stable for several months.
NAD[3]) 0.02M
Kept frozen in suitable portions
GK-suspension[3]) 1 mg/ml
Kept at 4° C.
GDH-suspension[3]) 10 mg/ml
Kept at 4° C.
Reagent-mixture
5.50 ml buffer; 0.20 ml ATP; 0.20 ml NAD; 0.04 ml glycerolkinase. Incubated for 15 minutes at 37° C immediately before use. This mixture should be used within 2 hours after its preparation.
Bloor-mixture: ethanol/diethylether 3:1 (V/V)
Silicagel Merck N° 7729 — particle size below 0.08 mm. Non-activated.
Chloroform
Ethanol
Ethanolic KOH-solution
a) stock solution (2%)
b) work solution (0.4%)
Acetic acid (6%)
Petroleum ether
Sulphuric acid 0.67N
Sodium hydroxide 0.67N

Glycerol stock solution: 400 µmol/liter
Prepared with glycerol p. a. Its water content is measured refractometrically.

Procedure

Collection of sample

Blood is drawn off in a centrifuge-tube containing 1 mg EDTA/ml blood. The plasma is immediately separated and deproteinised for the glycerol determination or extracted for the glyceride-glycerol determination.

Assay of glycerol

Transfer to a centrifuge tube in the order listed:

0.5 ml of aq. bidest; 1 ml of plasma; 1 ml of Zinc sulphate solution and 1 ml of 0.25N NaOH. Mix well and centrifuge after 5 minutes. Pipette the following into a cuvette: 0.5 ml of aq. bidest; 1.5 ml of reagent mixture and 1 ml of supernatant.
After incubation for 10 min. at 37° C, a first reading of optical density (E_1) against water is made at 340 nm. After adding 0.02 ml of GDH and incubation for 1 hour at room temperature, a second reading of optical density (E_2) is made.
A blank tube, in which 1 ml of aq. bidest. has been substituted for the supernatant, is prepared according to the same procedure as the unknown sample.

Calculation (3):

$$\frac{\Delta E}{0.062} \times 0.01 \times 3 \times 3.5 \times 1000 = \mu\text{mol glycerol/liter}$$

$\Delta E = \Delta E$ sample $- \Delta E$ blank

For concentrations higher than 200 µmol/liter, the supernatant should be diluted with aq. bidest. However the use of a standard curve elaborated with several dilutions of the glycerol standard solution is prefered to this formula. These dilutions are processed according to the same procedure as plasma.

Assay of glyceride-glycerol

Extract 0.75 ml of plasma with 14.25 ml of Bloor-mixture. Evaporate 10 ml of the extract, dissolve in 10 ml of chloroform and add 0.5 g of silicagel. Shake for 30 min. and evaporate 2 ml of the chloroform extract. Add 0.7 ml of ethanol and 0.3 ml of 0.4% ethanolic KOH solution to the residue. Simultaneously a blank is set up, with only 1 ml of ethanol added to the residue. Blank and sample are further treated in the same way. After heating for 30 min at 60° C; 0.1 ml of 6% acetic acid is added.
After evaporation in vacuo at room-temperature add 10 ml of petroleum ether and 5 min. later 1 ml of 0.67N sulphuric acid. Shake this mixture for 5 min., suck off and discard the petroleum ether. Add successively in a cuvette 0.9 ml of aq. bidest, 1.5 ml of the reagent mixture, 0.3 ml of the sulphuric acid layer (containing the free glycerol of the saponified glycerides), 0.3 ml of 0.67N NaOH. E_1 is measured after mixing and incubation for 10 min., at 37° C. E_2 is measured after adding 0.02 ml of GDH and incubation at room-temperature for 1 hour. The reagent blank is identical to the one used for the glycerol determination.

Calculation

$$\frac{\Delta E_1 - \Delta E_2}{0.062} \times 0.01 \times 3 \times 33.3 \times 1000 = \mu\text{mol glyceride-glycerol/liter}$$

$$\mu\text{mol glyceride-glycerol/liter} \times \frac{876^{4})}{1000 \times 10} = \text{mg/100 ml triglycerides.}$$

$\Delta E_1 = \Delta E$ after saponification $- \Delta E$ reagent blank
$\Delta E_2 = \Delta E$ without saponification $- \Delta E$ reagent blank

For concentrations over 1900 µmol/liter glyceride-glycerol or 165 mg/100 ml triglycerides the determination should be repeated, using 0.5 or 1 ml of chloroform extract. However the use of the same standard curve as used for the glycerol determination is indicated. The figure obtained on the absciss, multiplied with 9.5 gives µmol glyceride-glycerol/liter, and multiplied by 0.83, gives mg/100 ml triglycerides.

4) Average molecular weight of plasma triglycerides (3).

Results and Discussion

Deproteinising agent

Deproteinization with Zinc sulphate and NaOH does not result in values too low due to adsorption of glycerol to the precipitate or by inhibition of the enzymatic reaction. Indeed, whether the glycerol standard solution is treated with deproteinization-reagent or not, has no influence on the results obtained. However an excess of Zinc sulphate inhibits the enzymatic reaction. See table 1.

Tab. 1
The influence of deproteinising agent and excess Zinc sulphate on the enzymatic determination of glycerol. Glycerol standard solution, 200 µmol/l, ΔE without deproteinization = 0.115, titer NaOH = 0.250N

Titer Zinc sulphate solution (N)	ΔE
0.225	0.115
0.250	0.115
0.275	0.105
0.300	0.059
0.325	0.015

Incubation time

Incubation of the supernatant with the reagent mixture for more than 10 minutes is not necessary. Indeed the affinity of glycerolkinase for glycerol is such that the reaction comes to an end very quickly (2).
Deviation of the standard curve from the theoretical curve must be attributed to the inhibition of glycerophosphatedehydrogenase. The optical density reading over a 70 min. period proves that a maximum optical density is ensured, one hour after the addition of GDH. See figure 1.

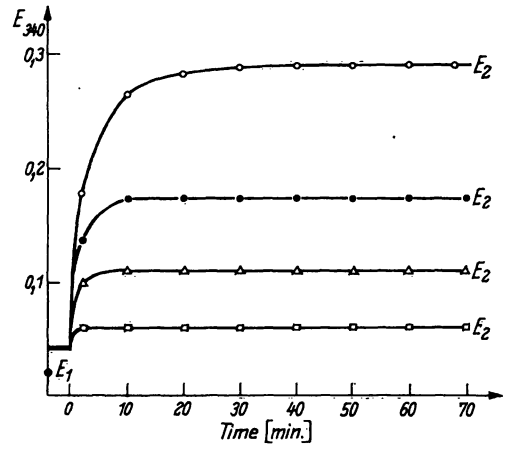

Fig. 1
Relationship of absorbance to time of incubation
o—o 400, •—• 200, △—△ 80 µmol glycerol/liter, □—□ blank

Stability of the reagent mixture

The reagent mixture is stable for 2 hours only. The older the reagent mixture, the higher E_1. Values for E_1, obtained with the reagent mixture freshly prepared, after 2 and after 5 hours storage, were respectively

0.032; 0.060 and 0.121. The final result with a 2 hours old reagent mixture, is still correct because E_2 increases to the same extent as E_1. But if an 5 hours old reagent mixture is used, the results are approximatively 15% too low.

With the aging of the reagent mixture a non enzymatic reaction presumably occurs between the hydrazine and NAD (8).

Standardisation

Comparison of the standard curve with the theoretical curve as calculated from the formula clearly indicates that the reaction is quantitative up to a concentration of 200 µmol/liter. For higher concentrations the standard curve should be used. See figure 2.

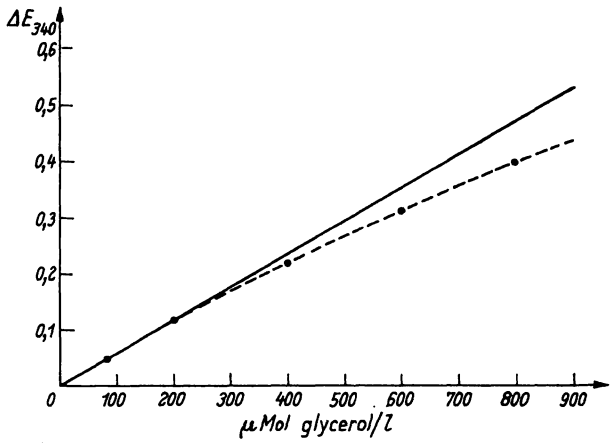

Fig. 2
Comparison of the standard curve •---• with the theoretical curve •—•

Specificity of the glycerol assay

Lactate does not interfere if GDH is not contaminated with lactic dehydrogenase. This can be checked by treating an aqueous solution of lactate by the same procedure as for the glycerol standard solution. With this technique the sum of glycerol and glycerol-1-phosphate is measured. L-α-glycerophosphate is not normally present in human plasma. But it appears after injection of fructose (1). However this metabolite can be determined separately by omitting glycerokinase from the reagent mixture.

Reproducibility

The reproducibility of the method evaluated by ten repeated analyses on one particular plasma specimen at a level of 158.8 µmol/liter resulted in a standard deviation of 3.9 µmol/liter.

The percentage recovery of glycerol added to plasma varies from 97 to 101%.

Stability of glycerol in deproteinised plasma

After deproteinization, the glycerol content does not change on storage. No differences were observed between determinations made immediately after deproteinization and those after storing the deproteinised sample for 3 days.

Glyceride-glycerol assay

The extraction of lipids, the removal of the phospholipids and the hydrolysis of the glycerides which preceed the enzymatic reaction, have been critically analysed in a previous publication (9). A tristearate solution of 120 mg/100 m*l* gave values of: 119.8; 122.1; 120.5; 117.7 and 120.3. The mean of ten determinations of glyceride-glycerol content performed on ten different samples, was 95.4 mg/100 m*l* for the colorimetric method (9) and 94.5 mg/100 m*l* for the enzymatic method.

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